Enhanced cutaneous vascular response in AD subjects under donepezil therapy

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Abstract

Objective: Abnormal cutaneous vasodilatory responses to the iontophoresis of vasodilators were previously observed in Alzheimer's disease (AD). We sought to replicate these observations and further identify peripheral vascular components of AD pathology.

Methods: Methacholine chloride (MCh), acetylcholine chloride (ACh), and sodium nitroprusside (SNP) were applied iontophoretically to forearm skin. Laser Doppler imaging of treated areas yielded total perfusion response values.

Results: Response to MCh was enhanced 78% ($P = 0.003$) in AD subjects under therapy with the acetylcholinesterase inhibitor (AChEI) donepezil ($N = 9$), relative to age- and sex-matched controls ($N = 12$). Significant increases in perfusion were also observed after application of ACh (68%, $P = 0.03$) and SNP (46%, $P = 0.04$).

Conclusions: A previous study reported attenuated response to ACh in AD. Paradoxically, we observed a substantially enhanced response that is likely a consequence of donepezil therapy. The increased response to the endothelium-independent vasodilator SNP indicates improved general vasodilatory response, perhaps due to preservation of endogenous ACh by donepezil. Cerebral perfusion in response to functional activation may be improved in this way, suggesting a secondary therapeutic mode of donepezil.
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1. Introduction

A significant body of evidence suggests that a systemic vascular pathology is present in Alzheimer's disease (AD). Altered endothelial function [1,12], blood pressure [22], and cutaneous active vasodilation [18] have been observed in AD. The apolipoprotein E ε4 (ApoE4) allele predisposes individuals to both atherosclerosis and AD [14]. Risk for dementia is increased fivefold in women who have experienced myocardial infarction [5]. Considerable evidence exists supporting the hypothesis that cerebral hypoperfusion due to microvascular dysfunction contributes to the genesis of AD [9,7].

Peripheral markers of AD are important not only in understanding the etiology of the disease but also for potentially improving the accuracy of the differential diagnosis of different types of dementia [13]. Such markers may possibly allow monitoring of the effects of therapeutic interventions.

Three previous studies have investigated the cutaneous dilatory response to iontophoresed vasoactive agents in AD. Hornqvist et al. [15] iontophoresed the α_1-agonist phenylephrine, the muscarinic agonist methacholine chloride (MCh), and the β_1-agonist isoproterenol and visually observed the resulting reddening or blanching of the skin. Only the response to isoproterenol was attenuated in the hospitalized subjects with severe AD studied. The experimental methods employed in this study involved the application of large iontophoretic currents that have subsequently been shown to directly affect vascular response, perhaps through the stimulation of perivascular nerves [4]. The results consequently require careful interpretation.

Algotsson et al. [1] iontophoresed sodium nitroprusside (SNP), isoproterenol, and acetylcholine chloride (ACh) and measured the dilatory response quantitatively using laser Doppler imaging. Significant reductions in response to isoproterenol and ACh were observed in the subjects with mild AD. Since ACh stimulates vascular endothelial cells to release nitric oxide (NO), the decreased response to this chemical is regarded as evidence of impaired endothelial function. The fact that the response to SNP was preserved indicates that the ability of smooth muscle to dilate under the influence

of NO was intact. A later study by Algotsson et al. found enhanced response to SNP in AD subjects harboring the ApoE4 allele over both controls and non-ApoE4 AD subjects [3].

In this paper, we attempt to replicate several of the results reported by Algotsson et al. in ref. [1].

2. Methods

2.1. Recruitment and selection of subjects

Subjects diagnosed with probable AD according to the National Institute of Neurological Disorders and Stroke (NINDS) criteria were recruited from the University of California at Davis Alzheimer's Disease Center. The control group was recruited from the spouses of the AD subjects and via press advertisement. Subjects were age- and sex-matched.

A total of 11 AD and 12 control (7 male and 5 female) subjects were examined.

Nine of the AD subjects (4 male and 5 female) were medicated with donepezil (dosage range of 0.06–0.19 mg/kg). We denote this group as the ADd group, and the medication-naive group (2 male and 1 female) as the ADn cohort.

Informed consent was obtained for all subjects under protocols approved by Institutional Review Boards at the participating institutions.

Subjects suffering from diabetes or hypertension (diastolic blood pressure >100 mmHg) were excluded [6].

None of the subjects regularly used tobacco products.

2.2. Iontophoresis solutions

Methacholine chloride, acetylcholine chloride, and sodium nitroprusside dihydrate were obtained and stored as dry matter (Fluka BioChemika AG, Buchs, Switzerland). ACh was maintained below 0 °C, MCh at 4 °C, and SNP at ambient temperature in the presence of a desiccant. All solutions used were prepared using deionized water within 2 h of each experiment and were stored in dark containers at room temperature before use.

2.3. Experimental procedure

All studies were performed between 09:00 and 11:30 a.m. to control for diurnal variation in endothelium-mediated vasodilation [11].

Subjects (patients and controls) were requested to fast and abstain from nicotine for 12 h before the study. Subjects were also asked to avoid the following medications for a specified number of days before the study: antioxidant vitamins (1 day), analgesics and anti-inflammatories (2 days), cyclo-oxygenase inhibitors and prostaglandins (2 weeks), adrenergic agonists and antagonists (1 day), sildenafil (1 day), monoamine oxidase inhibitors (3 weeks), inhaled nitrates/nitrites (1 day), steroids (3 weeks), antihistamines (2

days), and anticholinergic agents (1 day). We did not ask subjects to avoid statins as we assumed the effect of statins in restoring endothelial function was due to the long-term reduction of cholesterol levels, although recent evidence suggests that statins act directly on the endothelium to increase the synthesis of endothelial nitric oxide synthase (eNOS) and thus enhance vasodilatory response [19,24]. To address this issue, statin therapy is treated as a covariate in this study.

Subjects were instructed not to apply any ointments or skin lotions to their forearms on the day of the experiment.

After completion of the informed consent process, a fasting blood sample was taken from the right arm of all subjects for the purpose of assaying cholesterol and triglyceride levels.

Subjects were then seated in a temperature-controlled room (22 °C) for a period of 25 min in order to acclimate and relax after the blood draw. This acclamation period is necessary to avoid the confounding effects of constrictive vascular tone due to sympathetic activation induced by environmental stimuli, such as heat, cold, and psychological stress.

Blood pressure and pulse measurements were then taken on the arm that was not to receive iontophoretic treatment. The forearm to be examined was cleaned with a swab soaked in isopropanol and placed on an armrest under the scan area of a laser Doppler perfusion imager (Moor LDI, Moor Instruments, Devon, UK), and a baseline scan was taken of a 16.5 cm × 5.7 cm region of the dorsal forearm. To allow for simultaneous imaging of the response to the three chemicals, three iontophoresis chambers (ION-LDI, Moor Instruments) were placed along the axis of the forearm within the imaged area, as shown in Fig. 1.

Spatial reference points were marked on the skin using permanent marker at the outside radius of the annular cham-
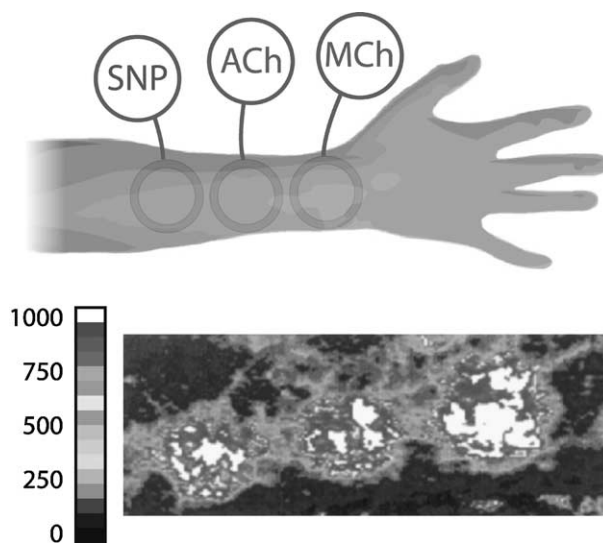

Fig. 1. The positioning of the iontophoresis chambers on the dorsal forearm is shown above a typical perfusion response to the iontophoresis of the vasoactive compounds. The color bar is scaled in arbitrary perfusion units.

bers at 45° to the axis defined by the centers of the chambers. The chambers were filled with ACh (0.1 mM), MCh (0.1 mM), and SNP (0.05 mM). At these concentrations, these chemicals elicit no vascular response in the absence of a driving current. An iontophoretic current of 110 μA was applied for 60 s to each chamber. The three currents were sourced by separate and isolated iontophoresis controllers (Moor MIC1-e, Moor Instruments) to avoid leakage currents. We have verified that the applied current density of 44 μA/cm^2 (110 μA/2.5 cm^2) does not induce measurable non-specific vasodilation, as has been reported at higher current densities [4].

Once drug delivery was complete, the chambers were removed and the affected skin was immediately scanned 10 times during the following 12 min.

Following the scan series, a second set of blood pressure and pulse measurements was taken.

3. Data analysis

Regions of interest (ROIs) were placed over the three response areas using custom digital image processing software. An image of typical perfusion response appears in Fig. 1. Delineation of the ROIs is an objective process based on the location of the spatial reference marks on the photographic scan. Since the LDI acquires the photographic and flux measurements simultaneously, these two scans are perfectly registered. We compensated for small interscan movements of the subjects' arms by aligning all of the scans based on the spatial reference points before placing the ROIs.

The mean of the 250 largest perfusion values within an ROI was taken for each of the three ROIs for each of the images to yield three 12-element time series. The sum of these time series provided three area-under-the-curve (AUC) values for each subject.

An ROI of 2.5 cm^2 (the inner area of the iontophoresis chamber) was placed over a typical area of the baseline scan of each subject. The mean flux within the baseline ROIs

was subtracted from the AUC curves before the data were analyzed.

ANOVA was applied individually to the three dependent variables AUC-MCh, AUC-ACh, and AUC-SNP. Membership of the ADd and control group served as the single factor in this analysis.

To establish whether donepezil dose influenced AUC-MCh, data from the ADd and ADn groups were pooled, and a regression analysis was performed.

ANCOVA was performed on the ADd data to control for the differences in diastolic blood pressure, body mass index (BMI), HDL cholesterol level, and triglycerides that were found during post hoc analysis of the data. The Wilk's Lambda likelihood ratio procedure was used to establish the significance of group differences ([20], p. 322).

All data processing and statistical analysis were performed using custom software developed under the Matlab 5.3 (The Mathworks, Natick, MA) environment.

4. Results

Table 1 summarizes the characteristics of the subjects examined while Fig. 2 illustrates the AUC-MCh versus the AUC-SNP values for the individual subjects. Owing to the similarity of the AUC-MCh and AUC-ACh values, the latter are not included in Fig. 2.

Comparison of the AUC values between the ADd and control groups revealed respective increases of 78% ($P = 0.003$), 68% ($P = 0.03$), and 46% ($P = 0.04$) in AUC-MCh, AUC-ACh, and AUC-SNP in the ADd group.

Regression analysis showed a moderate to weak negative correlation ($r = -0.34$) between body mass-normalized donepezil dose and AUC-MCh in the ADd group, but this correlation was not statistically significant ($P = 0.37$).

In the ADd group, there was a trend towards inverse correlation between AUC-MCh and Mini-mental State Examination scores ($r = -0.47$, $P = 0.12$). No such trend was observed for AUC-ACh or AUC-SNP.

Table 1
Summary of subject characteristics

Quantity	ADn group	ADd group	Controls
Number	3	9	12
Male/female	2/1	4/5	7/5
Age (years)	73.3 ± 3.2 (64, 83)	80.4 ± 0.5 (73, 88)	76.6 ± 0.5 (69, 84)
Body mass index (kg/m^2)	22.7 ± 3.4 (18.8, 25.1)*	24.0 ± 3.6 (18.3, 29.4)*	27.0 ± 3.1 (21.5, 33.9)
MMSE	24.3 ± 1.5 (20, 29)	18.9 ± 0.8 (2, 28)	N/A
Systolic BP (mmHg)	139.3 ± 2.0 (134, 146)	131.2 ± 1.9 (105, 158)	141.1 ± 1.5 (95, 159)
Diastolic BP (mmHg)	91.7 ± 1.7 (86, 96)	74.3 ± 1.0 (58, 85)**	86.8 ± 0.8 (76, 100)
Total cholesterol (mg/dl)	183.7 ± 13.4 (138, 213)	217.9 ± 4.6 (162, 267)	197.6 ± 3.1 (150, 259)
HDL cholesterol (mg/dl)	68.0 ± 6.6 (52, 90)	59.1 ± 1.7 (42, 87)	55.5 ± 1.4 (33, 82)
LDL cholesterol (mg/dl)	95.7 ± 8.6 (72, 123)	121.1 ± 3.8 (71, 172)	120.9 ± 2.6 (93, 178)
Triglycerides (mg/dl)	89.0 ± 14.5 (59, 139)	188.1 ± 9.9 (83, 333)*	109.1 ± 4.8 (53, 268)

Mean values are shown ±S.E.M. Interval bounds for the listed quantities appear in brackets. Quantities significantly different from controls are marked (*) for $P < 0.05$ and (**) for $P < 0.01$.
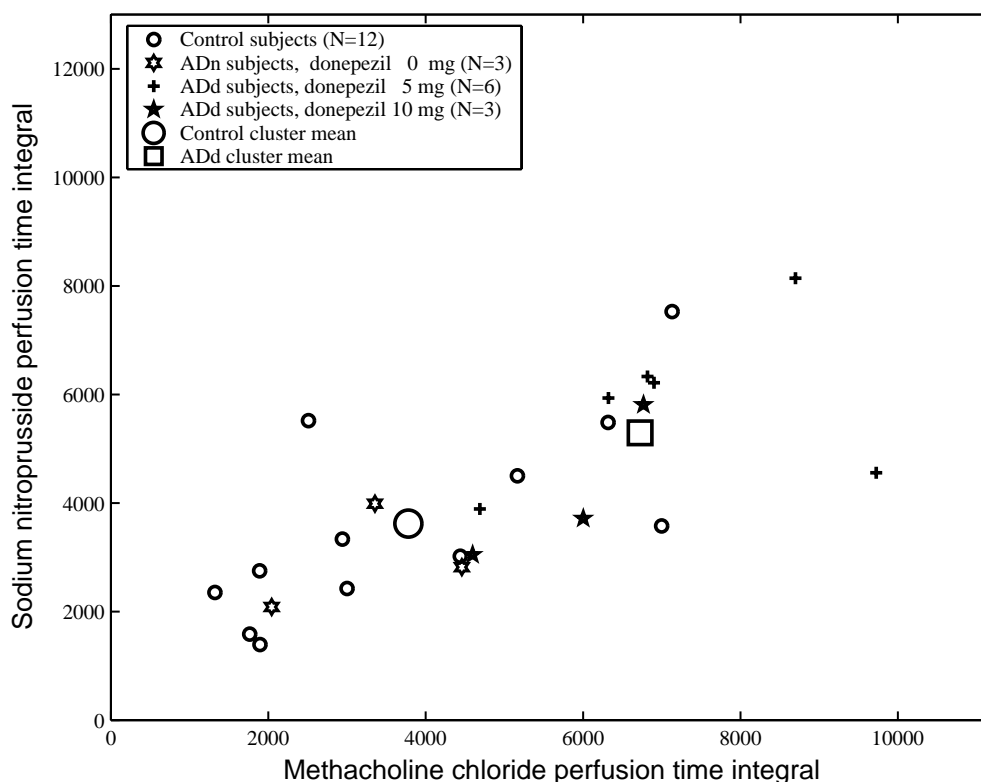
Fig. 2. Scatterplot showing AUC-SNP vs. AUC-MCh for all subjects. AUC-MCh is increased 78% ($P = 0.003$) and AUC-SNP 68% ($P = 0.03$) in the AD group under donepezil therapy, as compared to controls.

Mean ADd BMI was 11% lower than that of controls ($P = 0.05$). However, BMI did not correlate significantly with AUC-MCh in either the individual or pooled subject groups.

Triglyceride levels were much higher (72%, $P = 0.02$) in the ADd group relative to controls. Diastolic blood pressure was 14% lower in the ADd group with respect to controls ($P = 0.006$).

ANCOVA was performed in order to determine whether variables that significantly correlated with AUC-MCh could explain the observed differences in AUC-MCh between the control and ADd groups. With triglyceride level as a covariate, the significance is reduced from $P = 0.003$ to $P = 0.04$. We found a smaller reduction in significance when diastolic blood pressure is accounted for as covariate (from $P = 0.003$ to $P = 0.05$). ANCOVA that includes both diastolic blood pressure and triglyceride level as covariates completely "explains away" all significance of the difference in mean AUC-MCh between ADd and control groups.

A previous study found an inverse correlation between ACh and SNP perfusion responses and the LDL/HDL plasma ratio [4]. We observed no such correlations within individual groups or within the pooled subject population of our study.

Among men in the ADd group, the perfusion responses to MCh and ACh were strongly and significantly correlated with HDL level (AUC-MCh: $r = 0.97$, $P = 0.03$;

AUC-ACh: $r = 0.99$, $P = 0.01$). A previous study observed similar correlations in young and middle-aged women and not in men [2].

Two ADd subjects and four control subjects were under statin therapy. Since all available evidence suggests that statins enhance endothelial function, it is unlikely that statin therapy is a significant factor in this study given that AUC-MCh was so strongly enhanced in the ADd group relative to the control group.

Even though there were only three subjects in the ADn group, we have included these results in Fig. 2 for completeness.

5. Discussion

In the present study, we have observed large and significant enhancements of cutaneous vasodilatory response in AD subjects under donepezil therapy with respect to controls. In a previous study under a similar experimental protocol, impaired vasodilatory response to ACh has been reported in unmedicated AD subjects [1]. This previous study also found that the response to the endothelium-independent vasodilator SNP was preserved in AD.

We hypothesize that inhibition of acetylcholinesterase (AChE) by donepezil contributes towards these enhanced responses to cholinergic agonists by preventing the

hydrolysis of iontophoresed MCh and ACh by AChE. If this hypothesis is true, the well-tolerated, non-invasive measurement technique employed here could be pursued as a peripheral surrogate marker of AChE activity. This marker might be useful for monitoring and titrating drug therapy.

Determination of the optimal AChE inhibitor (AChEI) dose for a particular patient is complicated by the fact that cognitive benefit is sub-optimal when the AChEI dose is either too small or too large. An "inverted-U" dose–response characteristic appears to model several of these agents. In addition, the incidence and severity of side effects increase with dose [16]. Donepezil is a reversible inhibitor of brain and red blood cell (RBC) AChE [29]. Thus, the use of RBC AChE activity has been proposed as a surrogate marker for the effectiveness of donepezil. However, as carefully reviewed in ref. [28], RBC cholinesterase inhibition is not a reliable surrogate marker for the activity of AChEIs as a class of drugs, and its usefulness as a dose optimization tool for individual agents has yet to be clearly demonstrated. Invasively measured parameters, such as cerebrospinal fluid (CSF) concentrations of AChE, are increased approximately threefold in AD patients under donepezil therapy relative to unmedicated AD patients and controls. Increases in CSF-AChE concentration appear particularly pronounced in subjects who are clinically responsive to AChEIs, although the high variability of the CSF-AChE measurements decreases the significance of this correlation [8]. The requirement of regular lumbar punctures detracts from the attractiveness of this method for monitoring therapy. In further studies, we will attempt to ascertain whether the cutaneous vasoactivity measurements we have performed correlate with cognitive improvement and whether they constitute a more reliable marker than assays of RBC AChE activity.

Post hoc analysis of the data suggested possible confounding influences based on the observed significant differences in BMI, diastolic blood pressures, and triglyceride levels between the control and medicated AD groups. ANCOVA was applied to analyze the effects of these differences and correlations. The differences in the perfusion response between the control and ADd groups remained significant at the 95% confidence level when diastolic blood pressure, BMI, and triglyceride level were included as individual covariates, but not when these covariates were jointly considered. The results of the application of ANCOVA to this study should be treated with caution, however, since this method has a tendency to "explain away" true differences between group means ([20], pp. 339–349). This is especially true in the present case, where the regression model was designed to fit observed correlations that were not previously expected to exist in the data.

No previous studies have control for BMI and triglyceride levels. Algotsson found that elevated triglyceride levels decreased the perfusion response to ACh ($r = -0.54$, $P = 0.05$), SNP ($r = -0.70$, $P = 0.005$), and isoproterenol ($r = -0.66$, $P = 0.01$) in young and middle-aged women, but not in men [2]. Chronically, elevated levels of triglyc-

erides are associated with decreased forearm blood flow in response to the infused endothelium-dependent vasodilator serotonin and preserved response to ACh [10]. Assuming these observations apply in the cutaneous arterioles and accepting the results of Algotsson, this covariate would not be expected to contribute towards an enhanced AUC-ACh response measured in the ADd group, since these subjects had higher triglyceride levels.

The reasons why BMI might affect vasodilatory response are unclear. The integrity of endothelium-mediated vasodilation in the human brachial artery is not correlated with BMI [17,27], but it is unclear whether this is true in the microvasculature. It is possible that skin thickness, composition, and pore density are correlated with BMI, and that these factors affect the efficiency of iontophoretic delivery to the vascular endothelium and smooth muscle. Future studies should match subjects based on BMI, diastolic blood pressure, and triglyceride levels in order to avoid the need to treat these quantities as covariates in the analysis.

The large and significant differences between the responses of the ADd and ADn groups to all three agents are intriguing, but more medication-naive subjects are required in order to make any strong conclusions. Since most of our patient population receives AChEI therapy, unmedicated subjects are now uncommon.

Our data may provide an alternative explanation for the increased response to SNP observed previously in ref. [3] among AD subjects harboring the ApoE4 allele. Of the nine subjects in that study, four were under AChEI therapy. Among the ApoE4-negative group ($N = 8$), only one subject was medicated with an AChEI. It is quite possible that this large difference in the fraction of AD subjects taking medication contributed significantly to the observed difference in response between the groups. However, it is also possible that the enhanced response in ApoE4-positive subjects is due to membrane abnormalities and/or dysfunction of voltage-dependent ion channels [3].

The mechanism by which endothelium-independent vasodilation is enhanced in the ADd group relative to controls in response to the NO donor SNP is not clear. Martin et al. [21] demonstrated that local cholinergic mechanisms mediate NO-dependent vasodilation in vitro and that flow-mediated vasodilation is attenuated by AChE and atropine. It is widely accepted that when SNP enters the skin it releases NO, which diffuses into smooth muscle, causing relaxation and vessel dilation. Blood velocity increases as a consequence (as is directly confirmed by laser Doppler imaging of RBC flux), raising the shear stress induced by the blood on the endothelial cell walls. This increased shear stimulates the endothelium to release acetylcholine, which in turn leads to the release of more NO. In this way a decreased rate of hydrolysis of endothelium-derived acetylcholine might amplify the vasodilatory response of the arterioles to non-cholinergic vasodilators.

Our results suggest a possible mode of action by which donepezil increases cerebral blood flow in AD relative to

unmedicated controls [23]. Levels of circulating amyloid β (Aβ) are elevated in AD, and this major component of senile plaques has been shown to attenuate endothelium-dependent vasodilation in response to acetylcholine in cerebral blood vessels [26]. It is thus possible that enhancement of vascular response and partial reversal of the toxic effects of Aβ on endothelial cells by donepezil contribute to the cognitive improvement observed in patients treated with this AChEI. The preservation of cerebral blood flow may thus not stem solely from a slowing of neural degeneration, but may in fact contribute to this slowing. The availability of sufficient nutrient supply during functional activation is likely to be important for preservation of long-term neuronal function [25], and our results suggest a possible mechanism by which AChEIs enhance this supply. Agents, such as phosphodiesterase inhibitors, which prolong stimulated vasodilation via preservation of cGMP and/or cAMP in the vascular smooth muscle, would be expected to provide similar perfusion enhancement.

If the enhanced cutaneous vasodilation observed is indeed due to donepezil, this could help explain some adverse effects of this agent, such as vertigo and syncope.

The principal limitations of this study are that only a single experiment was performed on each subject and that significant differences in covariates were observed between the ADd group and controls. In future studies, we will attempt to match the groups in terms of these covariates. We will also attempt to determine:

1. Whether donepezil affects the perfusion response in control subjects.
2. Whether perfusion response correlates with the clinical efficacy of donepezil in AD subjects.
3. The effect of donepezil dosage changes on the perfusion response in an individual.
4. The correlation between perfusion response and RBC AChE assays.
5. The influence of ApoE genotype on perfusion response.
6. Whether the decline in the clinical efficacy of donepezil in individual patients correlates with a decline in the induced cutaneous perfusion response.

Acknowledgments

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